AMENDMENTS TO THE SPECIFICATION

Please replace paragraphs 0004, 0048, 0050, 0052, 0061, 0121, 0124, 0174, 0188, and 0201-0203 with the following amended paragraphs. Applicants note that the amendments, which are formal in nature, do not introduce new matter.

[0004] Although, with few exceptions, the genetic codes of all known organisms encode the same twenty amino acids, all that is required to add a new amino acid to the repertoire of an organism is a unique tRNA/aminoacyl-tRNA synthetase pair, a source of the amino acid, and a unique selector codon that specifies the amino acid (Furter (1998) Protein Sci., 7:419-426). Previously, we have shown that the amber nonsense codon, TAG, together with orthogonal *M. jannaschii* and *E. coli* tRNA/synthetase pairs can be used to genetically encode a variety ~~a variety~~ of amino acids with novel properties in *E. coli* (Wang et al., (2000) J. Am. Chem. Soc.,122:5010-5011; Wanget al., (2001) Science, 292:498-500; Wang et al., (2003) Proc. Natl. Acad. Sci. U. S. A.,100:56-61; Chin et al., (2002) Proc. Natl. Acad. Sci. U. S. A., 99:11020-11024), and yeast (Chin and Schultz, (2002) ChemBioChem, 3:1135-1137), respectively. The limited number of noncoding triplet codons, however, severely restricts the ultimate number of amino acids encoded by any organism.

[0048] **Figure 1** provides a sequence alignment of archaeal $tRNA^{Lys}$ sequences (SEQ ID NOs:1-23, from top to bottom). Genomic sequences derived from Pa, *Pyrococcus abyssi*; Pf, *Pyrococcus furiosus*; Ph, *Pyrococcus horikoshii*; Pya, *Pyrobaculum aerophilum*; Ta, *Thermoplasma acidophilum*; Tv, *Thermoplasma volcanum*; Af, *Archaeoglobus fulgidus*; Hh, *Halobacterium sp. NRC-1*; Mj, *Methanococcus jannaschii*; Mt, *Methanobacterium thermoautotrophicum*; Mm, *Methanosarcina mazei*; St, *Sulfolobus tokodaii*; Ss, *Sulfolobus solfataricus*; Ap, *Aeropyrum pernix* were aligned with the GCG program *pileup* and displayed with the program *prettybox*.

[0050] **Figure 3** schematically illustrates a consensus-derived amber suppressor tRNA (SEQ ID NO:24). The consensus for the family of archaeal $tRNA^{Lys}$ sequences is represented in a cloverleaf configuration. The anticodon loop was changed from the consensus to CUCUAAA to generate AK_{CUA}.

[0052] **Figure 5.** Construction of amber and four-base suppressor tRNAs. An amber suppressor tRNA (SEQ ID NO:24) was constructed from the multiple sequence alignments of many $tRNA^{lys}$ sequences. An Orthogonal AGGA suppressor tRNA was identified by selection from an acceptor stem library.

[0061] Translation systems that are suitable for making proteins that include one or more unnatural amino acids are described in International Publication Numbers WO 2002/086075, entitled "METHODS AND COMPOSITION FOR THE PRODUCTION OF ORTHOGANOL tRNA-AMINOACYL-tRNA SYNTHETASE PAIRS" and WO 2002/085923, entitled "IN VIVO INCORPORATION OF UNNATURAL AMINO ACIDS." In addition, see International Application Number PCT/US2004/011786, filed April 16, 2004. Each of these applications is incorporated herein by reference in its entirety. Such translation systems generally comprise cells (which can be non-eukaryotic cells such as *E. coli*, or eukaryotic cells such as yeast) that include an orthogonal tRNA (O-tRNA), an orthogonal aminoacyl tRNA synthetase (O-RS), and an unnatural amino acid (in the present invention, homoglutamine is an example of such an unnatural amino acid~~acod~~), where the O-RS aminoacylates the O-tRNA with the homoglutamine. An orthogonal pair of the invention includes an O-tRNA, e.g., a suppressor tRNA, a frameshift tRNA, or the like, and an O-RS. Individual components are also provided in the invention.

[0121] A composition of the invention includes an orthogonal aminoacyl-tRNA synthetase (O-RS), where the O-RS preferentially aminoacylates an O-tRNA with a homoglutamine. In certain embodiments, the O-RS comprises an amino acid sequence of PhKRS, E444G, PhΔAD, an I41 and/or S268 mutant of PhΔAD~~comprising SEQ ID NO.: 1~~,

or a conservative variation thereof. In certain embodiments of the invention, the O-RS preferentially aminoacylates the O-tRNA with an efficiency of at least 50% of the efficiency of an I41 and/or S268 mutation of PhΔAD~~a polypeptide comprising an amino acid sequence of SEQ ID NO.: 1~~.

[0124] A cell (e.g., a non-eukaryotic cell, or a eukaryotic cell) comprising a translation system is also provided by the invention, where the translation system includes an orthogonal -tRNA (O-tRNA); an orthogonal aminoacyl-tRNA synthetase (O-RS); and, a homoglutamine. Typically, the O-RS preferentially aminoacylates the O-tRNA with an efficiency of at least 50% of the efficiency of an I41 and/or S268 mutation of PhΔAD~~a polypeptide comprising an amino acid sequence of SEQ ID NO.: 1~~. The O-tRNA recognizes the first selector codon, and the O-RS preferentially aminoacylates the O-tRNA with the homoglutamine. In one embodiment, the O-tRNA comprises or is encoded by a polynucleotide sequence as set forth in SEQ ID NO.: 24 or SEQ ID NO.: 26~~SEQ ID NO.: 2~~, or a complementary polynucleotide sequence thereof. In one embodiment, the O-RS comprises an amino acid sequence of PhKRS, E444G, PhΔAD, an I41 and/or S268 mutant of PhΔAD~~as set forth in any one of SEQ ID NO.: 1~~, or a conservative variation thereof.

[0174] The incorporation of a homoglutamine or other unnatural amino acids can be done to, e.g., tailor changes in protein structure and/or function, e.g., to change size, acidity, nucleophilicity, hydrogen bonding, hydrophobicity, accessibility of protease target sites, target to a moiety (e.g., for a protein array), etc. Proteins that include a homoglutamine can have enhanced or even entirely new catalytic or physical properties. For example, the following properties are optionally modified by inclusion of a homoglutamine or other unnatural amino acid into a protein: toxicity, biodistribution, structural properties, spectroscopic properties, chemical and/or photochemical properties, catalytic ability, half-life (e.g., serum half-life), ability to react with other molecules, e.g., covalently or noncovalently, and the like. The compositions including proteins that include at least one homoglutamines are useful for, e.g., novel therapeutics, diagnostics, catalytic enzymes, industrial enzymes, binding proteins (e.g., antibodies), and e.g., the study of protein structure and function. *See,*

e.g., Dougherty, (2000) *Unnatural Amino Acids as Probes of Protein Structure and Function*, Current Opinion in Chemical Biology, 4:645-652. In addition, one or more unnatural amino acids can be incorporated into a polypeptide to provide a molecular tag, e.g., to fix the polypeptide to a solid support. *See e.g.*, "PROTEIN ARRAYS" by Wang and Schultz, filed December 22, 2003, international publication number WO2004/058946~~Attorney Docket Number 54-000810PC~~ for an extended discussion of methods of making arrays using polypeptides that comprise unnatural amino acids.

[0188] In order to produce antisera for use in an immunoassay, one or more of the immunogenic polypeptides is produced and purified as described herein. For example, recombinant protein can be produced in a recombinant cell. An inbred strain of mice (used in this assay because results are more reproducible due to the virtual genetic identity of the mice) is immunized with the immunogenic protein(s) in combination with a standard adjuvant, such as Freund's adjuvant, and a standard mouse immunization protocol (*see*, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a standard description of antibody generation, immunoassay formats and conditions that can be used to determine specific immunoreactivity. Additional details on proteins, antibodies, antisera, etc. can be found in USSN 60/479,931, 60/463,869, and 60/496,548 entitled "Expanding the Eukaryotic Genetic Code;" WO 2002/085923, entitled "IN VIVO INCORPORATION OF UNNATURAL AMINO ACIDS;" patent application entitled "Glycoprotein synthesis" filed January 16, 2003, USSN 60/441,450; and patent application entitled "Protein Arrays," USSN 60/435,821~~attorney docket number P1001US00~~ filed on December 22, 2002.

[0201] An orthogonal tRNA-synthetase pair derived from the type I lysyl-tRNA synthetase of *Pyrococcus horikoshii* has been developed for use in *E. coli*. The tRNA portion of the system functioned very well as an orthogonal amber suppressor. The synthetase-expression plasmid pKQ-PhE444G was able to charge this tRNA, but toxicity effects were still observed. When expressed alone, cells harboring pKQ-PhE444G grow to

56% of the density observed for cells with no plasmid._Reporter plasmids pAC-AK_{CUA} and pAC-AK_{CUA} show moderate toxicity as well~~, growing to 71% and 52%~~. When pKQ-PhE444G is cotransformed with plasmid pAC-AK_{CUA}, cell density is decreased to 17%. In addition, cells reach a density of only 5% when coexpressed with the β-lactamase reporter plasmid pAC-AK_{CUA} (a derivative of plasmid pACKO-A184TAG). It is therefore clear that there is toxicity with both the tRNA and the synthetase in this system. Furthermore, there appears to be a synergistic effect wherein cells cotransformed with both plasmids are drastically reduced in viability. To address this issue, we sought a less toxic mutant of PhKRS.

[0202] It was anticipated that point or other mutations in PhKRS might reduce the toxicity of the synthetase while retaining charging activity. Therefore, pKQ-PhE444G was transformed into chemically competent XL1-red cells (Stratagene) and the cells were plated on LB-agar plates containing 25 ug/mL kanamycin. This strain has several genomic mutations that cause a high rate of mutagenesis in transformed plasmids. Approximately 100 colonies were scraped from this plate and amplified in 25 mL of liquid LB media supplemented with kanamycin. It was anticipated that nontoxic mutants of pKQ-PhE444G would grow faster than the wild-type, and serial culture of the cells would lead to the accumulation of these mutants. After 2 serial cultures with 10000-fold dilution at each step, the cells were miniprepped and introduced into Genehog cells containing plasmid pAC-AK_{CUA} and plated on LB-agar plates containing 25 ug/mL each of kanamycin and chloramphenicol, and various concentrations of ampicillin. Greater than 90% of the transformed cells exhibited no apparent toxicity and were able to survive on LB-agar plates containing 1000 ug/mL ampicillin. Smaller colonies were observed even at 1500 ug/mL ampicillin indicating efficient amber suppression. One mutant synthetase, designated pKQ-PhKep, was isolated and characterized by restriction mapping and sequencing of the PhKRS open reading frame. The mutant gene contains an insertion of 778 bp following residue S357, but is otherwise the same sequence as plasmid pKQ-PhE444G. A BLAST search revealed that this insertion is homologous to a sequence annotated as "insAcp1" from plasmid p1658/97, but no other mention of this sequence has been observed in the literature,

and the source of this sequence is unknown. When translated from the start codon of PhKRS, the predicted product of this gene is truncated 6 amino acids downstream of S357. To test whether the truncation of PhKRS was responsible for the elimination of toxicity, primer CA510R with sequence 5'-CAGTGGAATTCAGTAAGTTGGCAGCATCAC-3' (SEQ ID NO:36) was synthesized to explicitly construct the truncation mutant in plasmid pKQ. Plasmid pKQ-PhKep was PCR-amplified with CA279 and CA510R, and the product was subcloned into the *Nco*I and *Eco*RI sites of plasmid pKQ. The resulting plasmid, pKQ-PhΔAD (also known as pKQ-Ph510), was cotransformed with plasmid pAC-AK_{CUA} and the resulting transformations ~~transformatations~~ were found to have a similar IC_{50} to pKQ-PhKep-transformed cells and no apparent toxicity.

[0203] The truncation after residue S357 appears to delete the anticodon binding domain of PhKRS, and we wanted to examine how this deletion affects the tRNA recognition properties of the synthetase. ~~Therfore~~Therefore, we overexpressed the synthetase ~~and~~ to perform aminoacylation assays *in vitro*. The gene was PCR-amplified from pKQ-PhKep with CA279 and CA511 (5'-CATTGGAATTCGAGTAAGTTGGCAGCATCAC-3', SEQ ID NO:37) and subcloned into the *Nco*I and *Eco*RI sites of pBAD-Myc/HisA in frame with the C-terminal Myc/His tag. Protein was purified by Ni-NTA chromatography.